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PRINCIPAL INVESTIGATOR: Timothy P. Quinn, M.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0962

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Timothy P. Quinn, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, San Francisco
San Francisco, California 94143-0962

E-Mail: tpquinn@itsa.ucsf.edu

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The purpose of this project is to use a novel chimeric cell death receptor (termed R2Fas) that is triggered by vascular endothelial growth factor (VEGF), as a means to kill prostate cancer ells and vascular endothelial cells.

The scope of this project involves: (i) creating adenoviral reagents to express the R2Fas receptor in prostate cancer cells and endothelial cells; (ii) determining if the R2Fas receptor kills cells in a VEGF-dependent manner; and (iii) identifying methods for increasing the killing activity of R2Fas.

The major findings to date include: (i) we have generated adenoviral reagents to express R2Fas as well as control adenoviruses; (ii) we have demonstrated that adenoviral-mediated expression of R2Fas in prostate cancer cells that overexpress VEGF activates apoptotic signaling and induces cell death; (iii) we have demonstrated that adenoviral-mediated expression of R2Fas in human endothelial cell is non-toxic, but renders the cells sensitive to killing when treated with VEGF; and (iv) we have demonstrated that R2Fas-mediated apoptosis can be potentiated by addition of several pharmacologic agents.

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INTRODUCTION

The purpose of this project is to use a novel chimeric cell death receptor (termed R2Fas) that is triggered by vascular endothelial growth factor (VEGF), as a means to kill prostate cancer cells and vascular endothelial cells.

The scope of this project involves: (1) creating adenoviral reagents to express the R2Fas receptor in prostate cancer cells and endothelial cells; (2) determining if the R2Fas receptor kills prostate cancer cells and vascular endothelial cells in a VEGF-dependent manner; and (3) identifying methods for increasing the killing activity of R2Fas.

The major findings to date include: (1) we generated adenoviral reagents to express R2Fas as well as three control adenoviruses; (2) we demonstrated that the adenoviral reagents direct expression of R2Fas or control receptors in DU145 prostate cancer cells and endothelial cells, and we confirmed expression of the receptors by immunoblot; (3) we demonstrated that expression of R2Fas in DU145 cancer cells activates apoptotic signaling and induces apoptosis in a very high percent of cancer cells within 72 hours; (4) we demonstrated that expression of R2Fas in endothelial cells does not induce apoptosis, but does render the cells sensitive to killing by VEGF treatment; and (5) we demonstrated that R2Fas-mediated apoptosis is potentiated by chemotherapeutic and pharmacologic agents.

BODY

Statement of Work

Task 1. Generate replication-defective adenovirus expressing R2Fas and appropriate control adenoviruses.

Task 1A: Subclone cDNAs for R2Fas and control receptors into adenoviral vector.
This task was accomplished in year 1.

Task 1B: Transfect adenoviral cDNA into 293A cells and recover adenovirus.
This task was accomplished in year 1.

Task 2. Use adenoviruses to express R2Fas or control viruses in prostate cancer cell lines and primary microvascular endothelial cells.

Task 2A: Transfect human prostate cancer cell lines and human microvascular endothelial cells in vitro with AdV/R2Fas or control adenoviruses. (Months 9-18)
Task 2B: Confirm that R2Fas protein is expressed in transfected cells by immunoblot techniques. (Months 9-18)

In the progress report for year 1 we showed that the AdV/R2Fas virus and AdV/CG-R2Fas control virus successfully directed expression of their respective receptors in DU145 prostate cancer cells and in porcine aortic endothelial cells. We have since demonstrated that both viruses direct expression of their receptors in human umbilical vein endothelial cells (HUVEC) (Figure 1, top panel). In this experiment DU145 cancer cells and HUVEC cells were infected with either AdV/R2Fas or AdV/CG-R2Fas at a multiplicity-of-infection (MOI, pfu/cell) of 40. Cells were lysed 48 hours after infection and immunoblotted with antibody against the hemagglutinin (HA) epitope tag present on the carboxyl tail of the receptors. As seen in Figure 1, R2Fas and the control receptor CG-R2Fas were expressed in both DU145 cells and HUVEC cells at similar expression levels. We are currently performing similar experiments with PC-3 and LNCaP prostate cancer cells, and human dermal microvascular endothelial cells.

Task 2C: For each prostate cancer cell line and endothelial cell type, characterize expression of VEGF, expression of apoptosis signaling or inhibiting proteins downstream of Fas, and sensitivity to endogenous Fas-mediated apoptosis. (Months 9-18)

We examined VEGF expression in DU145 cancer cells compared to normal human prostate epithelial cells (Figure 2). Cell lysates were immunoblotted with antibody against VEGF. As seen in Figure 2, DU145 cells over-express VEGF, which confirms published reports. Normal prostate epithelial cells do not have detectable VEGF expression.

We examined the expression levels of apoptosis signaling proteins in DU145 and PC-3 prostate cancer cells (Figure 3). Cell lysates were immunoblotted with antibodies against caspases-8, caspase-3, caspase-9, or VEGF. As seen in Figure 3, the expression level of these four proteins was similar in the two cancer cell lines. The equivalent expression of VEGF indicates that PC-3 cells, like DU145 cells, over-express VEGF compared to normal prostate epithelial cells. We are currently comparing the expression levels of other apoptosis mediators

and inhibitors, including FADD, FAP-1 (Fas associated phosphatase-1), FLIP, Bid, and XIAP. We will perform the same analysis for LNCaP cells.

Task 3. Determine response of prostate cancer cell lines to expression of R2Fas.

Task 3A: Beginning with prostate cancer cell lines that express high levels of VEGF and are sensitive to Fas-mediated apoptosis, determine if expression of R2Fas stimulates apoptosis, using standard apoptosis assays.

Using AdV/R2Fas virus, we demonstrated that expression of R2Fas in DU145 prostate cancer cells stimulated cleavage/activation of caspase-3 and caspase-9 (Figure 1). We previously demonstrated activation of caspase-8 and PARP cleavage (previous progress report). We then determined that expression of R2Fas directly stimulated apoptosis in DU145 cells (Figure 4). In this experiment DU145 cells were infected with AdV/R2Fas at various MOI (2.5 pfu/cell up to 20 pfu/cell), or with the control virus AdV/CG-R2Fas at MOI=20, or left uninfected. Cells were photographed daily. As seen in Figure 4, cells infected with the control virus remained normal and were not distinguishable from uninfected cells. In contrast, cells infected with AdV/R2Fas demonstrated cell fragmentation and cell death, beginning at 48 hours after infection. R2Fas expression induced cell death in a time-dependent and MOI-dependent manner, with virtually all cells killed by an MOI=20 at 72 hours. An MOI as low as 2.5 induced apoptosis in the majority of cells by 106 hours. These results demonstrate that DU145 prostate cancer cells can be killed by adenoviral-mediated expression of R2Fas.

To confirm that R2Fas-mediated apoptosis occurs via activation of caspases, we demonstrated that the pancaspase inhibitor ZVAD blocks R2Fas-mediated apoptosis (Figure 5). In this experiment DU145 cells were infected with AdV/R2Fas at an MOI of 10 or 20, in the presence or absence of ZVAD (40 μM). Cells were photographed and lysed at 48 hours after infection. Lysates were immunoblotted with antibody against cleaved/activated caspase-3. As seen in Figure 5 (top panel), caspase-3 activation induced by R2Fas expression was completely blocked by ZVAD. The lower panel of Figure 5 demonstrates that ZVAD blocked cell death as well, confirming that cell death is mediated via caspase activation.

To prove that R2Fas-mediated apoptosis in DU145 cells is due to VEGF over-expression (and not due to expression of a Fas domain-containing transgene), we initially attempted to use RNAi to knock down VEGF expression levels in DU145 cells. However, we were unable to significantly change VEGF protein levels even with prolonged and repeated transfection of anti-VEGF RNAi reagents. Therefore we took the opposite approach and demonstrated that further increasing VEGF expression in DU145 cells potentiated R2Fas-mediated apoptosis (Figures 6-8). DU145 cells were infected with AdV/R2Fas (MOI=10) plus either AdV/VEGF (MOI=10) or AdV/LacZ (MOI=10). Cells were lysed 32 hours after infection, and immunoblotting was performed. As seen in Figure 6, R2Fas expression was similar in cells co-infected with VEGF or LacZ (top panel), and VEGF expression was increased in cells infected with AdV/VEGF (middle panel). The bottom panel demonstrates that increased expression of VEGF stimulated increased cleavage/activation of caspase-3. Figure 7 shows a similar experiment in which DU145 cells were infected with AdV/R2Fas + AdV/VEGF, or AdV/R2Fas + AdV/LacZ. Additional controls were cells infected with AdV/LacZ or AdV/VEGF. As seen in Figure 7, cells co-expressing R2Fas and VEGF apoptosed at a significantly higher level than cells expressing R2Fas alone.

In a further experiment, we compared cell death in DU145 cells co-infected with AdV/R2Fas and AdV/VEGF, but in this experiment we varied the order of infection (Figure 8). The first

group was infected with both viruses simultaneously (or R2Fas + LacZ virus as control). The second group was infected with AdV/R2Fas on day 1, and AdV/VEGF or AdV/LacZ on day 2. The third group was infected with AdV/VEGF or AdV/LacZ on day 1, and AdV/R2Fas on day 2. When examined at 72 hours, all cells that were co-infected with AdV/R2Fas + AdV/VEGF showed increased cell death compared to cells infected with AdV/R2Fas + AdV/LacZ, and the effect was most pronounced in cells infected with AdV/VEGF simultaneous with or after AdV/VEGF. These experiments demonstrate that increased expression of VEGF in DU145 cells potentiated R2Fas-mediated apoptosis. These results rule out the possibility that R2Fas-mediated apoptosis is due to non-specific activation of Fas domain-mediated apoptosis.

We have begun to examine the response of PC-3 cells to R2Fas expression (Figure 9). PC-3 cells were infected with AdV/R2Fas or AdV/CG-R2Fas control virus at an MOI=30 or 60 and photographed at 56 and 128 hours. Cells expressing R2Fas were decreased in number compared to uninfected cells or cells expressing the control receptor CG-R2Fas (Figure 9). We are currently determining if the decrease in cell number represents cell death or decreased proliferation. We are also examining the response of LNCaP prostate cancer cells.

We examined the response of normal human prostate epithelial cells to R2Fas expression, and hypothesized that these cells would be resistant to killing by R2Fas expression because they do not express VEGF (see Figure 2). Cells were infected with AdV/R2Fas or the control virus AdV/CG-R2Fas at an MOI of 10 or 20. As seen in Figure 10, both receptors were expressed in normal human prostate epithelial cells. Notably, these cells do not undergo apoptosis when R2Fas is expressed (Figure 11). This result is important because it suggests that R2Fas-mediated apoptosis is restricted to cells that overexpress VEGF. We will confirm this by using AdV/VEGF to force expression of VEGF in normal human prostate epithelial cells, and then determine if they are killed by expression of AdV/R2Fas.

Task 4. Determine response of endothelial cells to expression of R2Fas.

Task 4A: Confirm that expression of R2Fas in the absence of VEGF does not kill endothelial cells.

In the progress report for Year 1 we showed that adenoviral-mediated expression of R2Fas in porcine aortic endothelial cells did not stimulate apoptosis. We have extended these results to HUVEC cells (Figure 12). HUVEC cells were infected with AdV/R2Fas or the control AdV/CG-R2Fas at an MOI=40. Expression of the receptors is seen in the top panel of Figure 1. Expression of R2Fas had no apparent effect on the growth or morphology of HUVEC cells, and specifically did not induce apoptosis (third panel, Figure 12). Expression of R2Fas in HUVEC did produce slight cleavage/activation of caspase-3 (Figure 1, middle panel), but no activation of caspase-9 (Figure 1, lower panel). These results demonstrated that HUVEC cells, which do not express VEGF, can express R2Fas at high levels without toxicity if VEGF is not present.

Task 4B: Determine if expression of R2Fas renders endothelial cells sensitive to killing by VEGF.

Our hypothesis is that expression of R2Fas in endothelial cells will cause them to die by apoptosis when treated with VEGF. We found that HUVEC cells expressing R2Fas did in fact undergo apoptosis when treated with VEGF (Figure 12, right panel). In this experiment, HUVEC cells were infected with AdV/R2Fas at an MOI=40. One hundred hours after infection cells expressing R2Fas appeared normal. Cells were then treated with VEGF-165 (1 nM for 24

hours). As seen in the right panel, VEGF treatment induced cell death and fragmentation. This demonstrates that expression of R2Fas renders endothelial cells killable by VEGF treatment.

A likely scenario for a preclinical test of R2Fas as an anti-angiogenic therapy would involve injection of AdV/R2Fas into a tumor. In this setting, R2Fas expression in endothelial cells would occur in a tissue microenvironment already containing VEGF secreted by the tumor. This is different from the experiments we have done in vitro, in which AdV/R2Fas infection and subsequent R2Fas expression occur in the absence of VEGF, and VEGF is then added later. To better approximate the in vivo situation, we asked if AdV/R2Fas expression would still induce apoptosis in endothelial cells if VEGF was already present in the cell culture medium (Figure 13). In this experiment porcine aortic endothelial cells were infected with AdV/R2Fas at an MOI=20 in medium containing escalating levels of VEGF-165, from 0 nM to 1 nM. We found that expression of R2Fas induced apoptosis when VEGF was present at 1 nM. This result more closely approximates an in vivo setting, and indicates that R2Fas expression can stimulate endothelial apoptosis when VEGF is continuously present.

Task 5. Determine if pharmacologic agents and co-expression of apoptosis effector proteins potentiate R2Fas-mediated apoptosis.

Task 5A:Treat prostate cancer cells and endothelial cells expressing R2Fas with IFNγ, TNFα, cisplatin, actinomycin D, camptothecin or BIS VIII to determine if these agents potentiate the apoptotic activity of R2Fas.

One way to enhance the apoptotic activity of R2Fas is to add pharmacologic agents that may potentiate Fas-mediates apoptotic signaling. We screened six agents on DU145 cells to determine the highest concentration of each drug that did not induce overt cell toxicity. We then combined that drug concentration with AdV/R2Fas infection to determine if the drug potentiated R2Fas-mediated apoptosis. We specifically chose drug concentrations that did not kill DU145 cells on their own so that we would be able to detect enhanced R2Fas-mediated apoptosis. The drug concentrations we used were:

BisVIII	1 μM
Camptothecin	0.05 μM
Etoposide	0.5 μM
Taxol	0.5 nM
Doxorubicin	1.0 μg/mL
Cisplatin	1.0 μg/mL

DU145 cells were pretreated for 12 hours with each of the drugs at the indicated concentrations, and then infected with AdV/R2Fas at an MOI=10. (This relatively low MOI was chosen so that potentiation by the drugs could be detected. In preliminary experiments, a higher MOI induced apoptosis so rapidly that the drug effects were difficult to determine). As seen in Figure 14, all six agents were non-toxic at the concentrations used (top photos). All six agents potentiated R2Fas-mediated apoptosis compared to cells that received R2Fas and vehicle (bottom photos). The extent of apoptosis potentiation was quite remarkable, with virtually complete cell death in cells expressing R2Fas and treated with camptothecin or doxorubicin. These findings are important because they suggest that combining non-cytotoxic drug treatment

with R2Fas expression will greatly enhance R2Fas apoptotic activity. We are currently performing similar experiments with PC-3, LNCaP, and microvascular endothelial cells.

Task 5B: Use adenovirus-mediated gene transfer to co-express R2Fas with downstream effector caspases caspase-8 or caspase-3 in prostate cancer cells and endothelial cells. Determine if co-expression makes cells more sensitive to killing by VEGF.

We have received the cDNA encoding caspase-8, and we will soon purchase the cDNA encoding caspase-3. We will use these cDNAs to generate adenoviruses expressing their respective proteins. We will then determine if R2Fas-mediated apoptosis can be potentiated by increased expression of these effector caspases.

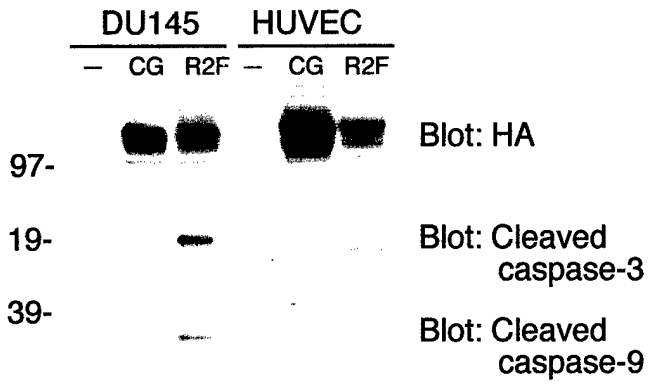


Figure 1. Adenoviral-directed expression of R2Fas receptor in DU145 prostate cancer cells and endothelial cells. DU145 prostate cancer cells and human umbilical vein endothelial cells (HUVEC) grown in 6-well plates were infected with AdV/CG-R2Fas or AdV/R2Fas at MOI = 40. Cells were lysed 48 hours after infection and lysates were electrophoresed by SDS/PAGE. Immunoblotting was performed with: HA antibody to visualize receptor expression (top panel); antibody against cleaved, active caspase-3 (middle panel); or antibody against cleaved, active caspase-9 (lower panel). Compared to DU145 prostate cancer cells, expression of R2Fas in endothelial cells stimulated much less activation of caspase-3 and caspase-9.

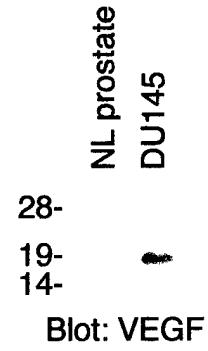


Figure 2. Overexpression of VEGF in DU145 prostate cancer cells. Lysates of normal prostate epithelial cells or DU145 prostate cancer cells were electrophoresed by SDS-PAGE and immunoblotted with antibody against VEGF.

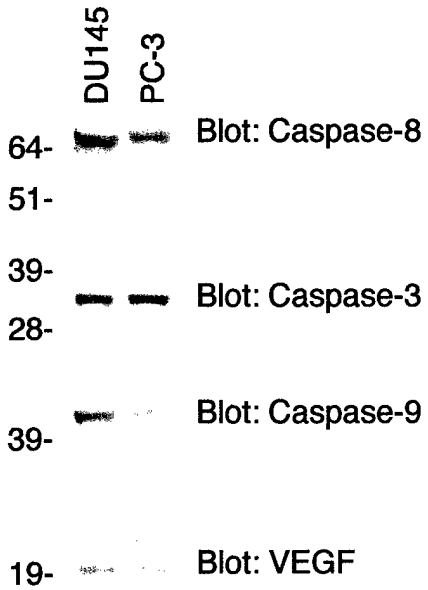


Figure 3. Expression of apoptosis proteins in DU145 and PC-3 prostate cancer cells. DU145 cells and PC-3 cells were grown in 6-well plates, lysed, and equal amounts of lysate protein were electrophoresed by SDS/PAGE. Immunoblotting was performed with antibodies to: Caspase-8, Caspase-3, Caspase-9, or VEGF. The two prostate cancer cell lines express similar level of these proteins.

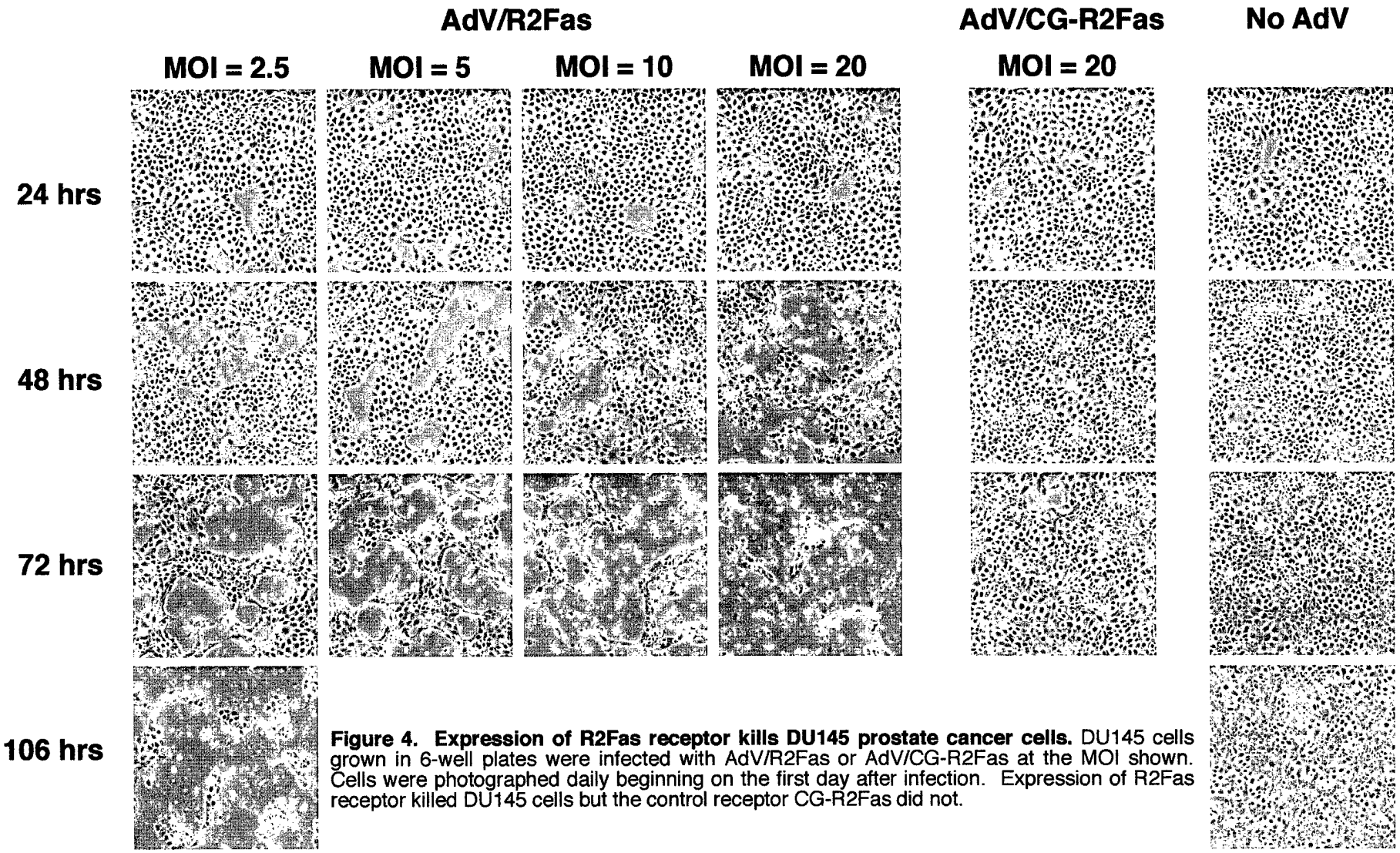


Figure 4. Expression of R2Fas receptor kills DU145 prostate cancer cells. DU145 cells grown in 6-well plates were infected with AdV/R2Fas or AdV/CG-R2Fas at the MOI shown. Cells were photographed daily beginning on the first day after infection. Expression of R2Fas receptor killed DU145 cells but the control receptor CG-R2Fas did not.

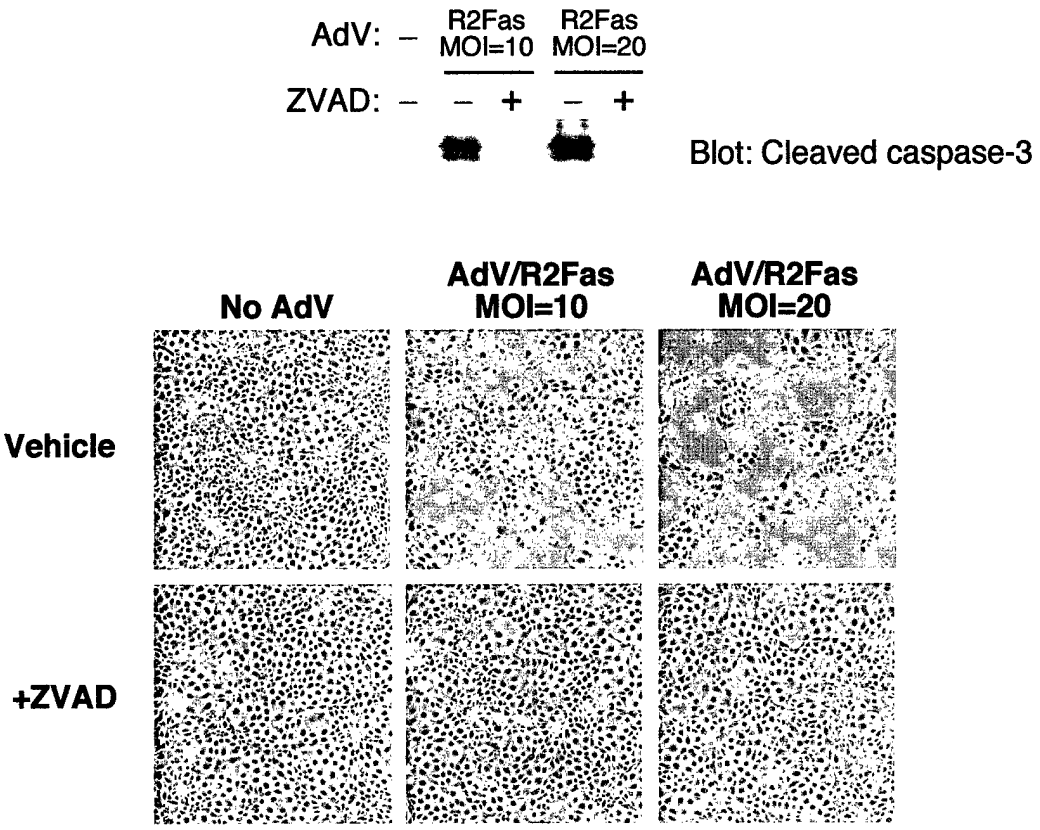


Figure 5. ZVAD blocks R2Fas-mediated caspase activation and cell death. DU145 cells grown in 6-well plates were infected with AdV/R2Fas at the MOI shown in the presence of ZVAD (40 μM) or vehicle (DMSO). Cells were photographed and lysed 48 hours after infection. Lysates were electrophresed by SDS/PAGE and immunoblotted with antibody against cleaved caspase-3 (top panel). The pancaspase inhibitor ZVAD blocked caspase-3 activation (top panel) and blocked cell death (lower panel), confirming that R2Fas expression stimulates apoptosis via caspase activation.

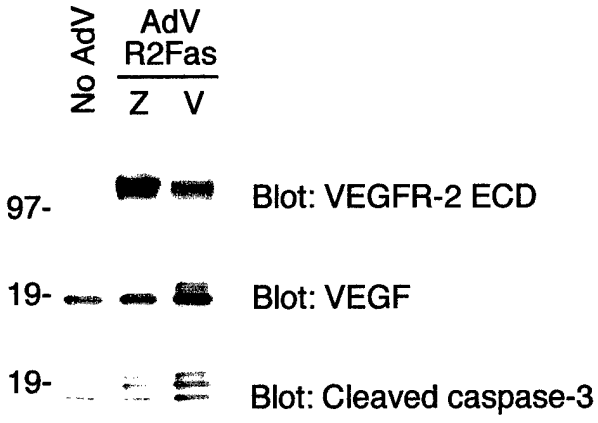


Figure 6. Co-expression of VEGF-165 potentiates R2Fas-mediated caspase-3 activation. DU145 cells grown in 6-well plates were infected with AdV/R2Fas at MOI=10 (lanes 2 and 3), and co-infected with either AdV/LacZ at MOI=10 (lane 2) or AdV/VEGF-165 at MOI=10 (lane 3). Cells were lysed 32 hours after infection. Increased expression of VEGF-165 by AdV/VEGF is seen in lane 3 in the middle panel. The lower panel shows increased caspase-3 cleavage in cells over-expressing VEGF.

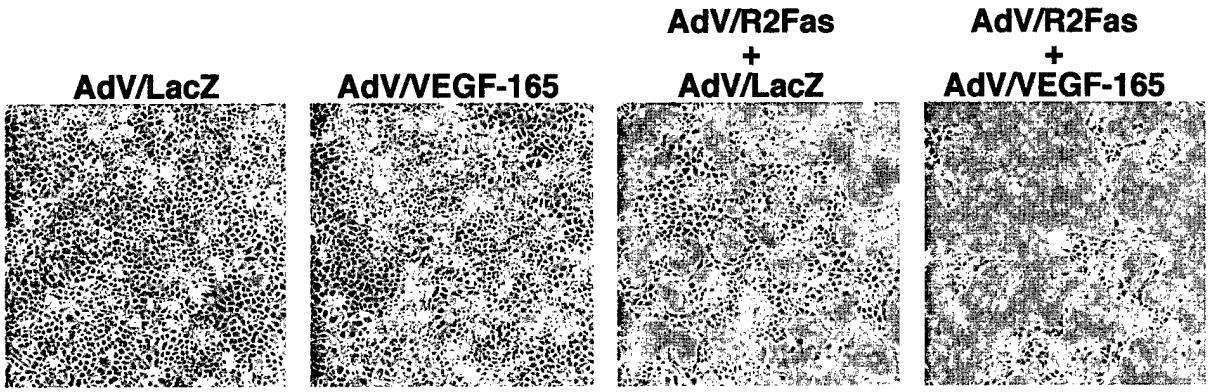


Figure 7. Co-expression of VEGF-165 potentiates R2Fas-mediated cell death. DU145 cells grown in 6-well plates were infected with either: AdV/LacZ alone (MOI=40); AdV/VEGF alone (MOI=40); AdV/R2Fas plus AdV/LacZ (MOI=20 for each); or AdV/R2Fas plus AdV/VEGF (MOI=20 for each). Cells were photographed 96 hours after infection. Co-infection of AdV/VEGF increased cell death mediated by expression of R2Fas, confirming that R2Fas activity responds to the level of VEGF expression.

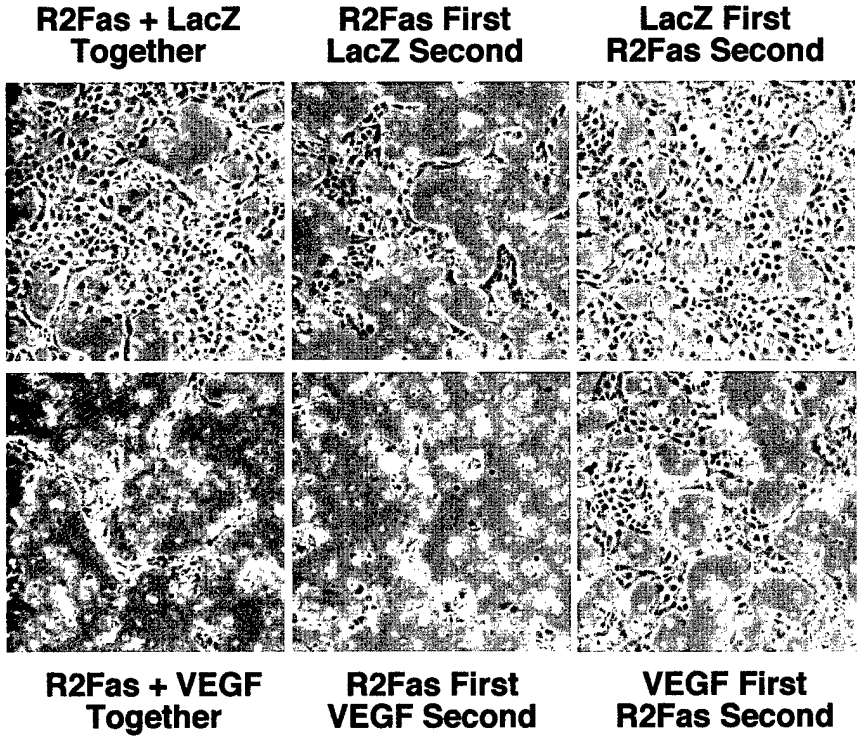


Figure 8. Co-expression of VEGF-165 potentiates R2Fas-mediated cell death. DU145 cells grown in 6-well plates were infected with AdV/ R2Fas (MOI=10) plus either AdV/LacZ (MOI=10) or AdV/VEGF (MOI=10). Cells on left were infected at the same time with both viruses. Cells in center panel were infected with AdV/R2Fas 24 hours before AdV/LacZ or AdV/VEGF. Cells on right were infected with AdV/LacZ or AdV/VEGF 24 hours before AdV/R2Fas. Increased expression of VEGF potentiated R2Fas-mediated apoptosis, especially when VEGF expression was simultaneous with or after R2Fas expression (left and center panels).

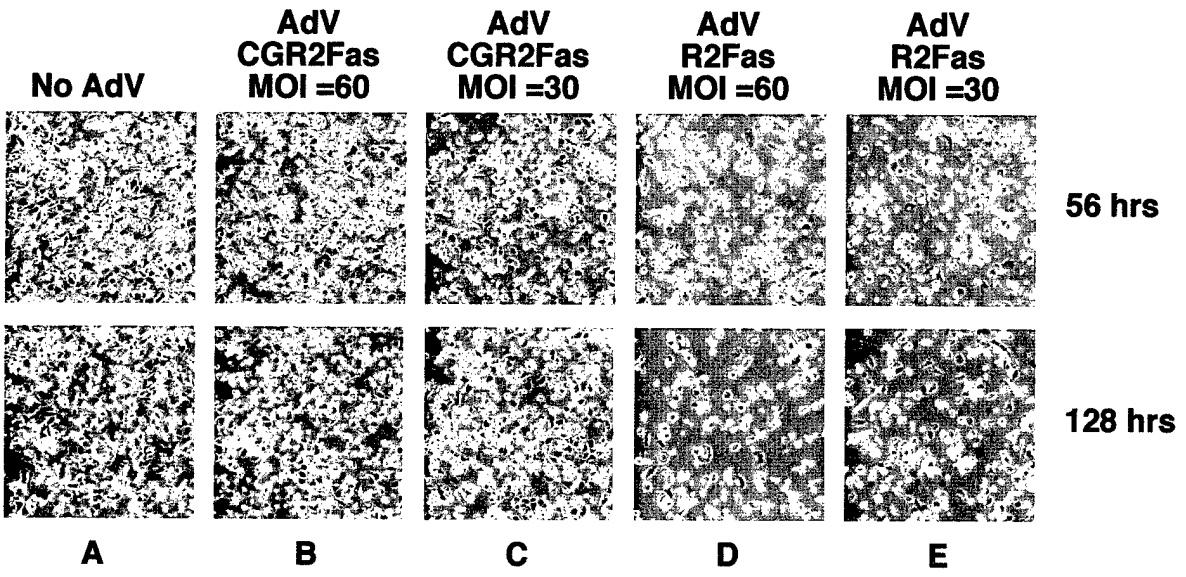


Figure 9. Expression of R2Fas receptor decreases PC-3 cell numbers. PC-3 cells grown in 6-well plates were infected with AdV/CG-R2Fas or AdV/R2Fas at the MOI indicated. Cells were photographed 56 and 128 hours after infection. Expression of R2Fas led to fewer total cells compared to expression of the control CG-R2Fas receptor (D vs. B, or E vs. C). We are investigating whether this is due to apoptosis or decreased cell proliferation.

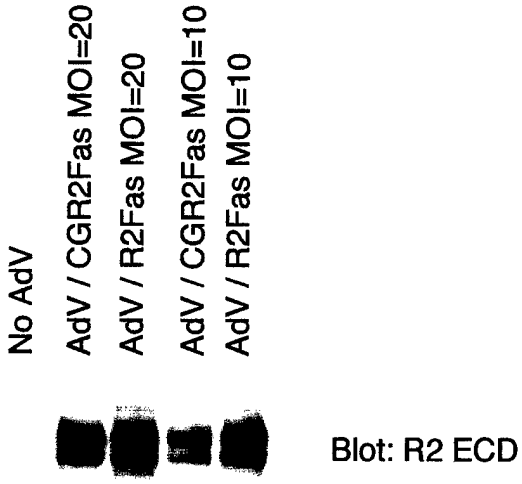


Figure 10. Expression of R2Fas receptor or control CG-R2Fas receptor in normal prostate epithelial cells. Normal primary human prostate epithelial cells grown in 6-well plates were infected with AdV/R2Fas or AdV/CG-R2Fas at MOI=10 or MOI=20 as indicated. Cells were lysed 48 hours after infection and lysates were electrophoresed by SDS/PAGE. Immunoblotting was performed with an antibody against the VEGFR-2 extracellular domain to visualize receptor expression.

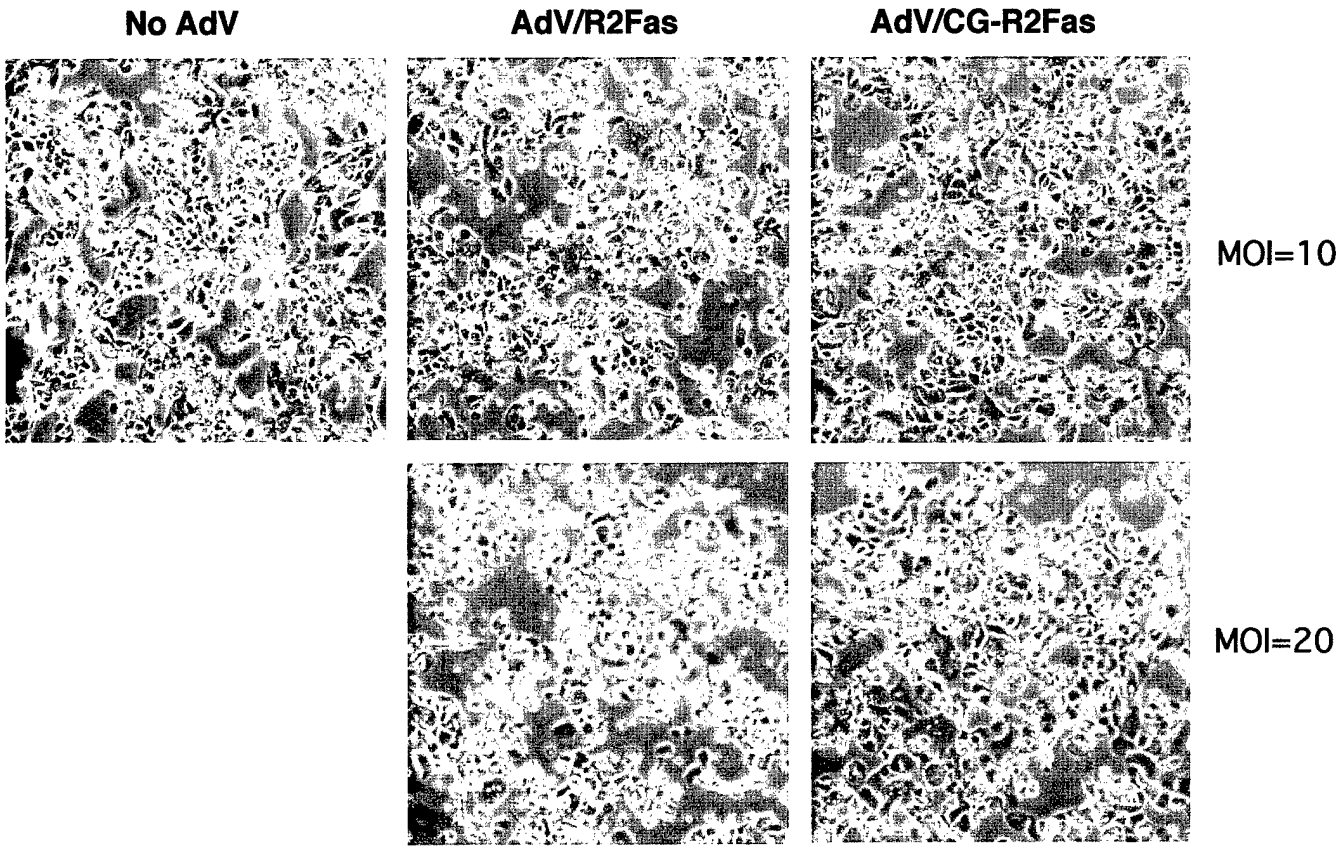


Figure 11. Expression of R2Fas does not stimulate apoptosis in normal human primary prostate epithelial cells. Normal human primary prostate epithelial cells grown in 6-well plates were infected with AdV/R2Fas or AdV/CG-R2Fas at MOI=10 or MOI=20 as indicated. Cells were photographed 72 hours after infection. Unlike DU145 prostate cancer cells, expression of R2Fas in normal prostate cells did not stimulate apoptosis.

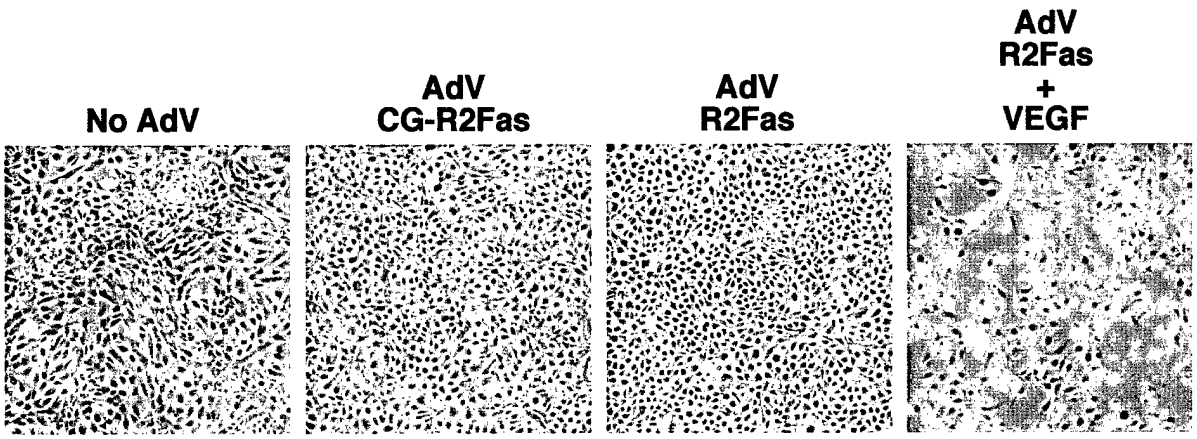


Figure 12. Expression of R2Fas receptor does not kill HUVEC cells but renders them killable by VEGF. Human umbilical vein endothelial cells (HUVEC) grown in 6-well plates were infected with AdV/CG-R2Fas or AdV/R2Fas at MOI = 40. Unlike DU145 cells, expression of R2Fas did not kill HUVEC cells (third panel; 100 hours after infection). However, R2Fas expression rendered HUVEC cells sensitive to killing by VEGF treatment (right panel, 1 nM VEGF-165 X 24 hours).

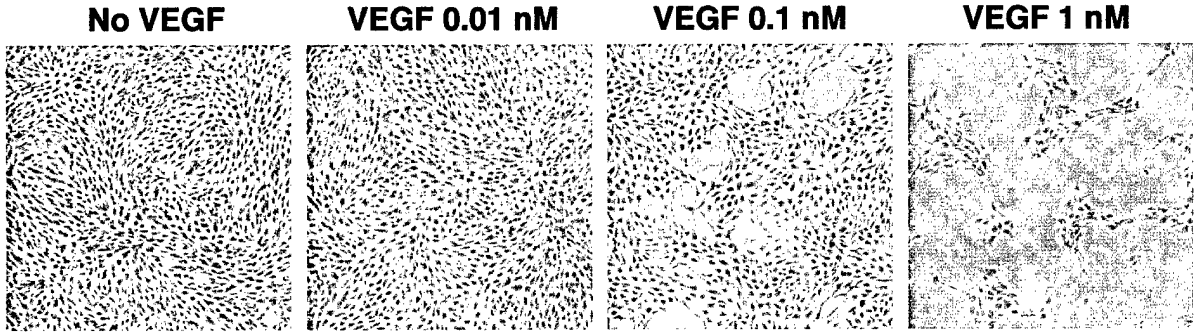


Figure 13. Expression of R2Fas receptor in the presence of VEGF kill endothelial cells. Porcine aortic endothelial cells were infected with AdV/R2Fas (MOI=20) in media containing no VEGF-165 (left panel) or the indicated concentrations of VEGF-165. Cells were photographed 96 hours after infection. Expression of R2Fas in cells treated with VEGF at 1 nM stimulated apoptosis (right panel).

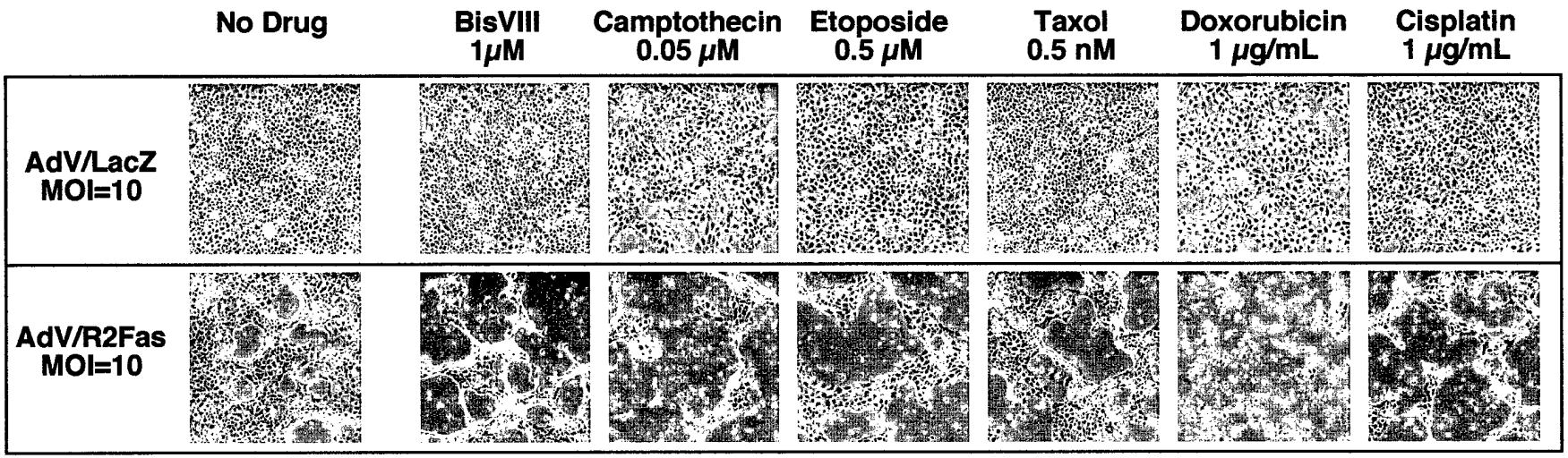


Figure 14. Potentiation of AdV/R2Fas-mediated cell death by pharmacologic agents. DU145 cells grown in 6-well plates were pretreated with the indicated agents for 12 hours before infection with AdV/LacZ or AdV/R2Fas at an MOI=10. Cells were photographed 36 hours after infection. All agents potentiated R2Fas-mediated apoptosis, especially BisVIII, camptothecin, etoposide, doxorubicin, and cisplatin.

KEY RESEARCH ACCOMPLISHMENTS

a. Created adenoviruses that express the R2Fas death receptor, and control adenoviruses that express the inactive control receptors CG-R2Fas or LacZ.
b. Demonstrated adenoviral-directed expression of R2Fas in DU145 prostate cancer cells and in endothelial cells.
c. Demonstrated that expression of R2Fas in DU145 cancer cells activates apoptotic signaling in a time- and titer-dependent manner.
d. Demonstrated that expression of R2Fas, but not the control receptor CG-R2Fas, stimulates apoptosis in DU145 prostate cancer cells.
e. Demonstrated that R2Fas-mediated apoptosis is potentiated by treatment of cells with: BisVIII, camptothecin, etoposide, taxol, doxorubicin, and cisplatin.

REPORTABLE OUTCOMES

a. The adenoviruses expressing R2Fas and CG-R2Fas will be shared with other interested investigators.
b. An abstract and poster describing this work was presented at the American Association for Cancer Research - NIH - EORTC International Conference on Molecular Targets and Cancer Therapeutics, November 2003 in Boston.
c. Our work was highlighted in a press release and press conference from the AACR Conference.
d. An abstract and poster describing this work was presented at the Keystone Conference on Angiogenesis: Novel Basic Science Insights and Human Therapy, January 2004 in Santa Fe.
e. Funding applied for based on work supported by this award:
   DOD Breast Cancer Research Program, Concept Award, submitted 1/2004
   DOD Ovarian Cancer Research Program, New Investigator Award, submitted 2/2004
   DOD Prostate Cancer Research Program, Idea Award, submitted 2/2004

CONCLUSIONS

In summary, we have generated adenoviral reagents that direct expression of the VEGF-triggered death receptor R2Fas, and the inactive control receptor CG-R2Fas. We have used these adenoviral reagents to express R2Fas and the control receptor in DU145 prostate cancer cells and human endothelial cells, and we have demonstrated that expression of R2Fas activates caspase signaling. Most importantly, we have found that expression of R2Fas in DU145 cells stimulates extensive killing. This may be the first demonstration that a growth factor can be forced to act as a death factor by activating an engineered receptor. We have further found that expression of R2Fas in endothelial cells does not induce apoptosis, but does render the cells sensitive to killing by treatment with VEGF. These findings suggest that expression of R2Fas in tumor cells and tumor blood vessels may be a feasible and novel form of combined tumor and antiangiogenesis therapy.

REFERENCES

APPENDICES

1. Poster presented at the American Association for Cancer Research - NIH - EORTC International Conference on Molecular Targets and Cancer Therapeutics, November 2003 in Boston.
2. Poster presented at the Keystone Conference on Angiogenesis: Novel Basic Science Insights and Human Therapy, January 2004 in Santa Fe.
3. Press release from the AACR conference highlighting our work.

Killing cancer cells and endothelial cells with a VEGF-triggered cell death receptor

Tim Quinn, Carlos San Mateo and Adrian Padron
University of California San Francisco
tpquinn@itsa.ucsf.edu 415-502-5196

AACR - NIH - EORTC International Conference
Molecular Targets and Cancer Therapeutics
November 17-21, 2003

ABSTRACT

Vascular endothelial growth factor (VEGF-A) is a dimeric angiogenic factor that is overexpressed by many cancer cells. VEGF produced by tumors is a major mediator of tumor angiogenesis. Consequently, numerous approaches to inhibit VEGF expression or activity are under investigation as anti-angiogenic cancer therapies, including neutralizing antibodies, soluble receptors, ligand traps, small molecule kinase inhibitors, and RNA-targeted agents. While some agents have recently been shown to have efficacy in clinical trials, it is too early to know if anti-VEGF therapies will prove to be highly effective, free of resistance, and without significant side effects.

Given the central role of VEGF in tumor angiogenesis, we have investigated an alternative approach that is not based on inhibition of VEGF activity, but instead converts VEGF from an angiogenic growth and survival factor into a death factor. In essence our goal is to turn tumor-derived VEGF into a weapon against the tumor itself and/or its blood vessels.

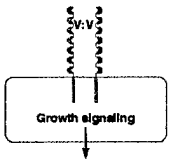
We created a chimeric receptor (termed VEGFR2Fas) by fusing the extracellular, VEGF-binding domain and the transmembrane domain of VEGFR-2 (also known as Flk-1/KDR) to the cytoplasmic "death domain" of the Fas apoptosis receptor (CD95/Apo-1). As a control, we also generated an inactive receptor with no apoptotic activity (CG-VEGFR2Fas) by incorporating a previously described inactivating point mutation into the Fas death domain (lprcg mutation).

Using retroviral or adenoviral gene transfer techniques we expressed VEGFR2Fas or the control CG-VEGFR2Fas receptor in endothelial cells and cancer cell lines in vitro. When expressed in porcine aortic endothelial (PAE) cells, VEGFR2Fas had no apoptotic effect in the absence of VEGF. When VEGF was added, cells were rapidly killed by activation of caspase-mediated apoptotic signaling. Cells expressing the control CG-VEGFR2Fas receptor were unaffected by exposure to VEGF. These results demonstrate that VEGFR2Fas is a VEGF-triggered cell death receptor. Further investigation demonstrated that VEGF stimulated formation of hetero-oligomeric receptor complexes composed of VEGFR2Fas and Neuropilin-1 (Np-1), a non-kinase receptor for VEGF. Engagement of Np-1 into a complex with VEGFR2Fas significantly potentiated the apoptotic activity of VEGFR2Fas, which was demonstrated using a peptide that blocked binding of VEGF to Np-1, and by comparing the relative apoptotic activities of different VEGF isoforms that do or do not bind to Np-1.

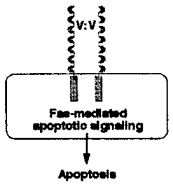
To determine if expression of VEGFR2Fas induced apoptosis in cancer cells that overexpress VEGF, we used adenovirus to transiently express VEGFR2Fas or the control CG-VEGFR2Fas in DU145 human prostate cancer cells. DU145 cells infected with AdV/VEGFR2Fas (MOI ~10-40) began to express VEGFR2Fas by 24 hours and underwent caspase-mediated apoptosis beginning at 48 hours. Expression of the control CG-VEGFR2Fas receptor had no effect.

Our results demonstrate that VEGF can be transformed from a growth factor into a cell death factor by engaging the artificial chimeric death factor VEGFR2Fas along with the Np-1 receptor. Further investigation will identify how this effect can be optimized and will determine the feasibility of using this approach in vivo to directly kill tumor cells and/or their blood vessels.

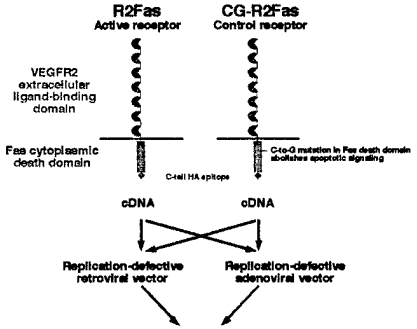
Normal VEGF signaling



Hypothesis:
Can VEGF be made into a death factor by engaging a chimeric receptor composed of VEGFR-2 and Fas?



METHODS



Endothelial cells: HUVEC, Porcine aortic
DU145 prostate cancer cells
MDA-MB-231 breast cancer cells

Confirm expression of R2Fas or CG-R2Fas receptors
Determine if cells endogenously express VEGF
?Does expression of R2Fas directly kill cells
?Does expression of R2Fas make cells killable by exogenous VEGF
Apoptosis assays

Methodology:

Cells were grown in 6-well plates in their respective medium which included fetal bovine serum (2% FBS for HUVEC and 10% FBS for all others). Retroviral transfection was used to generate porcine aortic endothelial cells (PAE) stably expressing R2Fas or CG-R2Fas. Stable cell lines were generated by selection in G418 (250 μg/mL). For adenoviral experiments, when cells were ~50% confluent one well was trypsinized to determine cell number, and an appropriate volume of titered AdV preparation was added to generate a specific multiplicity-of-infection (MOI). Cells were lysed and Western blotting performed using SDS-PAGE under reducing conditions.

Reagents:

cDNAs:
R2Fas consists of the entire mouse VEGFR-2 (Flk-1) extracellular and transmembrane domains fused to the entire cytoplasmic domain of human Fas (CD95/Apo-1) receptor, with a hemagglutinin epitope tag on the carboxyl tail (Quinn et al, Can. Res., 61:8628, 2001).

CG-R2Fas contains a C-to-G point mutation in the Fas death domain (lprcg mutation) that abolishes apoptotic signaling (Quinn et al, Can. Res., 61:8628, 2001). The CG-R2Fas mutation was generated by PCR-directed mutagenesis and was provided by Dr. Kevin Ramer.

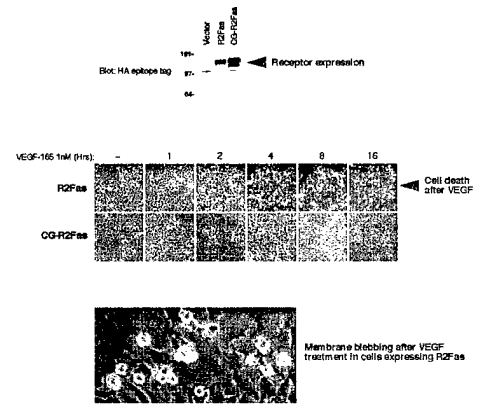
Np-1 cDNA was generously provided by Dr. Shi-Yuan Cheng, University of Pittsburgh.

Cells:
HUVEC: from Clonetics
DU145 and MDA-MB-231: from UCSF cell culture facility

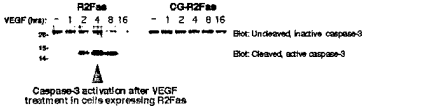
Replication-defective retroviral vector: LNCX (CMV promoter, neomycin resistance; Clontech)
Retroviral packaging cells: PA317
Replication-defective adenoviral vector: Adeno-X (CMV promoter, Type 5 ΔE1/ΔE3; Clontech)
Adenovirus titer: Clontech AdV titer kit
Antibodies:
Caspase and PARP antibodies: Cell Signaling Technology
Np-1: Oncogene Research
VEGF: Santa Cruz and Pharmingen
Growth factors: VEGF-165, VEGF-121, PlGF from R&D Systems
Chemicals: ZVAD from Biomol
Np-1 blocking peptide (PlGF exon 9): RRPKGRGKRRRENGRP from UCSF peptide facility

RESULTS

VEGF kills porcine aortic endothelial cells expressing the R2Fas receptor

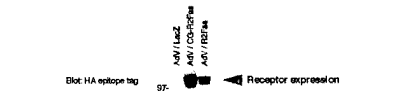


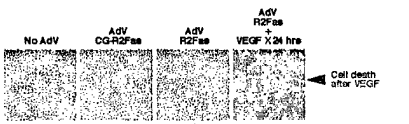
Membrane blebbing after VEGF treatment in cells expressing R2Fas



Caspase-3 activation after VEGF treatment in cells expressing R2Fas

VEGF kills human umbilical endothelial cells expressing the R2Fas receptor

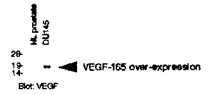




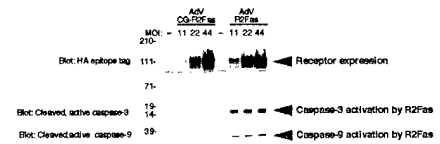
No apoptosis in absence of VEGF

RESULTS

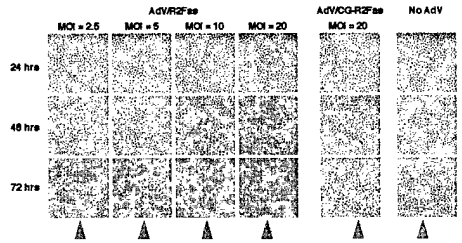
DU145 prostate cancer cells overexpress VEGF-165



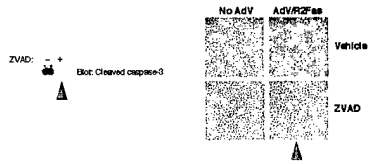
R2Fas expression in DU145 cells activates apoptotic signaling



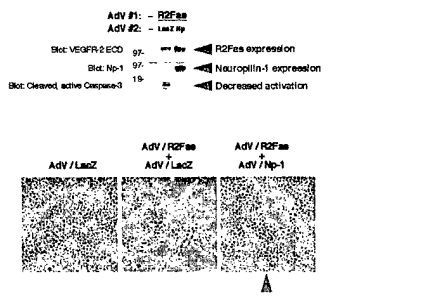
R2Fas expression kills DU145 cells



ZVAD caspase inhibitor protects DU145 cells from R2Fas-induced apoptosis



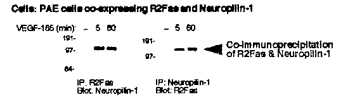
Over-expression of Neuropilin-1 (another VEGF receptor) protects DU145 cells from R2Fas apoptosis



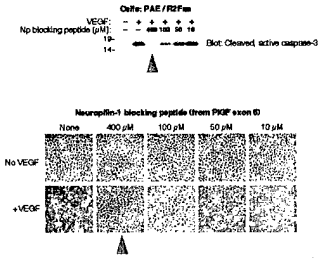
Inhibition of R2Fas-mediated apoptosis by co-expression of a second VEGF receptor (Neuropilin-1) demonstrates that R2Fas-mediated apoptosis is due to endogenous VEGF expression.

RESULTS

R2Fas forms a complex with Neuropilin-1 in a VEGF-dependent manner

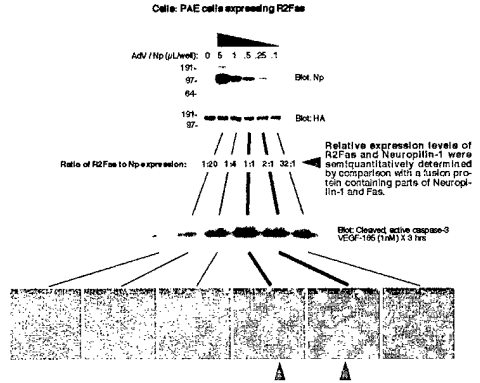


A peptide that blocks VEGF binding to Neuropilin-1 inhibits R2Fas-mediated apoptosis



Inhibition of R2Fas-mediated apoptosis by a Neuropilin-1 blocking peptide indicates that Neuropilin-1 contributes to the apoptotic activity of R2Fas.

Neuropilin-1 potentiates the activity of R2Fas when expressed at equivalent levels of expression, but over-expression of Neuropilin-1 inhibits R2Fas



Maximal caspase-3 activation and cell death occurs when R2Fas and Neuropilin-1 expression are approximately equivalent.

SUMMARY

The chimeric receptor R2Fas combines the extracellular and transmembrane domains of VEGFR-2 with the cytoplasmic death domain of the Fas apoptosis receptor.

VEGF kills endothelial cells (HUVEC or PAE) expressing R2Fas. No apoptosis occurs in the absence of VEGF.

Therefore R2Fas is a VEGF-triggered death receptor.

Cells that over-express VEGF (e.g. DU145 prostate cancer cells) are killed by R2Fas expression.

R2Fas stimulates apoptosis by activating Fas-mediated caspase signaling.

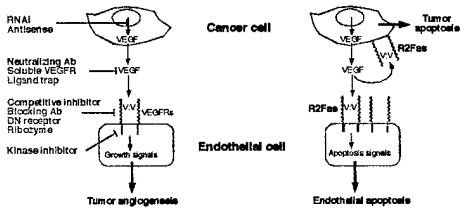
R2Fas forms a complex with the Neuropilin-1 receptor in a VEGF-dependent manner. Neuropilin-1 potentiates the apoptotic activity of R2Fas when expressed at similar levels.

IMPLICATIONS

These results suggest a new approach to cancer therapy in which VEGF produced by cancer cells is made to act as a death factor for the cancer cells themselves and/or their blood vessel cells.

Current anti-angiogenesis techniques based on VEGF/VEGFR inhibition

Anti-tumor and anti-angiogenesis approach based on R2Fas



Compared to conventional anti-angiogenesis approaches that focus on inhibiting VEGF/VEGFR expression or activity, a method based on R2Fas may have unique advantages:

- **Targets both the tumor and its vessels**
- **Kills instead of inhibiting growth**
- **Low toxicity to normal cells that express or are exposed to low levels of VEGF**
- **Increased VEGF expression in ischemic tumor and stromal cells would likely enhance R2Fas activity**

Killing cancer cells and endothelial cells with a VEGF-triggered cell death receptor

Tim Quinn, Carlos San Mateo and Adrian Padron
University of California San Francisco
tpquinn@itsa.ucsf.edu (415) 502-5196

Keystone Conference
Angiogenesis: Novel Basic Science Insights and Human Therapy
January 13-18, 2004

ABSTRACT

Vascular endothelial growth factor (VEGF-A) is a dimeric angiogenic factor that is overexpressed by many cancer cells and is a major mediator of tumor angiogenesis. Consequently, numerous approaches to inhibit VEGF expression or activity are under investigation as anti-angiogenic cancer therapies, including neutralizing antibodies, soluble receptors, ligand traps, small molecule kinase inhibitors, and RNA-targeted agents. It is not yet known if anti-VEGF therapies will prove to be highly effective, free of resistance, and without significant side effects.

Given the central role of VEGF in tumor angiogenesis, we have investigated an alternative approach that is based not on inhibition of VEGF activity, but instead converts VEGF from a growth and survival factor into a death factor. In essence our goal is to turn tumor-derived VEGF into a weapon against the tumor itself and/or its blood vessels.

We created a chimeric receptor (VEGFR2Fas) by fusing the extracellular, VEGF-binding domain and the transmembrane domain of VEGFR-2 to the cytoplasmic "death domain" of the Fas apoptosis receptor (CD95/Apo-1). As a control, we generated a receptor with no apoptotic activity (CG-VEGFR2Fas) by incorporating a previously described inactivating point mutation into the Fas death domain (lprog mutation).

Using retroviral or adenoviral vectors we expressed VEGFR2Fas or the control CG-VEGFR2Fas receptor in endothelial cells and cancer cell lines in vitro. When expressed in porcine aortic endothelial (PAE) cells, VEGFR2Fas had no apoptotic effect in the absence of VEGF. But when VEGF was added, cells were rapidly killed by activation of caspase-mediated apoptotic signaling. Cells expressing the control receptor were unaffected by VEGF. These results demonstrate that VEGFR2Fas is a VEGF-triggered cell death receptor. Further investigation showed that VEGF stimulated formation of hetero-oligomeric receptor complexes composed of VEGFR2Fas and Neuropilin-1 (Np-1), a non-kinase receptor for VEGF. Engagement of Np-1 into a complex with VEGFR2Fas significantly potentiated the apoptotic activity of VEGFR2Fas.

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Supported by grants from American Cancer Society and DOD (DAMD17-02-1-0029).

Hypothesis:
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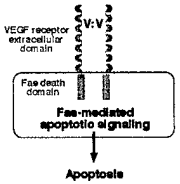
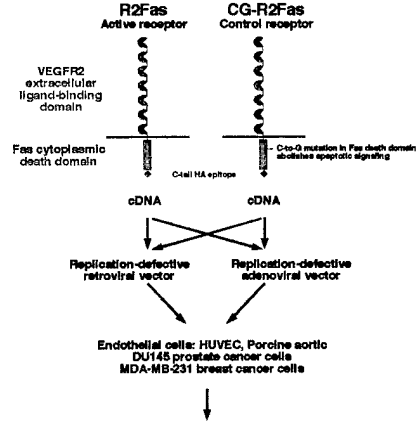


METHODS



Methodology:

Cells were grown in 6-well plates in their respective medium which included fetal bovine serum (2% FBS for HUVEC and 10% FBS for all others). Retroviral transduction was used to generate porcine aortic endothelial cells (PAE) stably expressing R2Fas or CG-R2Fas. Stable cell lines were generated by selection in G418 (250 μg/mL). For adenoviral experiments, when cells were ~50% confluent one well was trypsinized to determine cell number, and an appropriate volume of titered AdV preparation was added to generate a specific multiplicity-of-infection (MOI). Cells were lysed and Western blotting performed using SDS-PAGE under reducing conditions.

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Np-1 cDNA was generously provided by Dr. Shi-Yuan Cheng, University of Pittsburgh.

Cells:
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DU145 and MDA-MB-231: from UCSF cell culture facility

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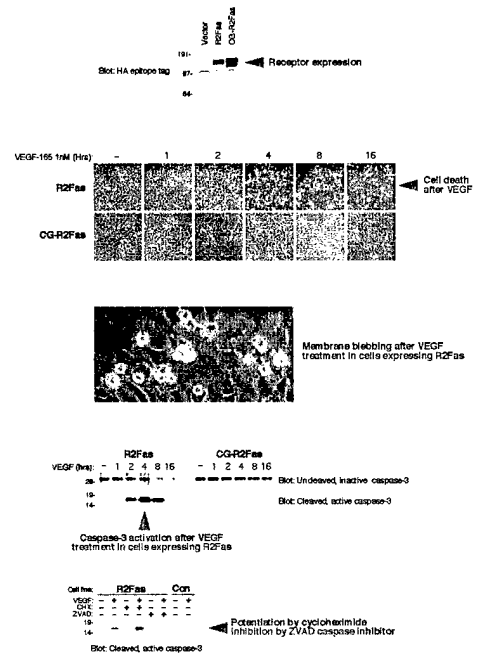
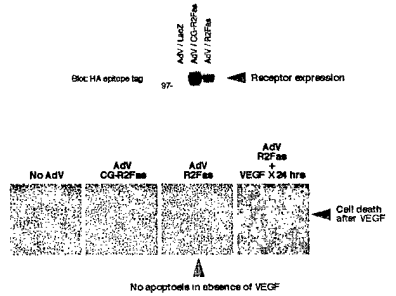
Caspase and PARP antibodies: Cell Signaling Technology
Np-1: Oncogene Research
VEGF: Santa Cruz and Pharmingen
Growth factors: VEGF-165, VEGF-121, PlGF from R&D Systems
Chemicals: ZVAD from Biomol
Np-1 blocking peptide (PlGF exon 6): RRPKGRGKRRRENGRP from UCSF peptide facility

RESULTS

1. VEGF kills porcine aortic endothelial cells expressing the R2Fas receptor

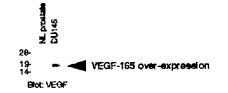


2. VEGF kills human umbilical endothelial cells expressing the R2Fas receptor

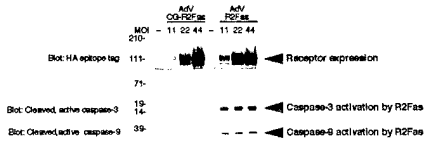


RESULTS

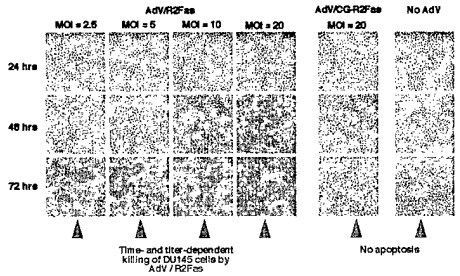
3. DU145 prostate cancer cells overexpress VEGF-165



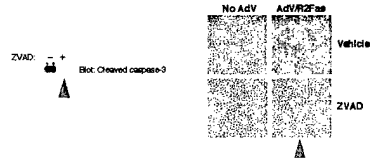
4. R2Fas expression in DU145 cells activates apoptotic signaling



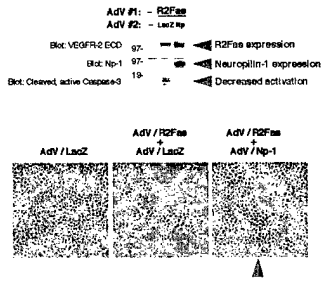
5. R2Fas expression kills DU145 cells



6. ZVAD caspase inhibitor protects DU145 cells from R2Fas-induced apoptosis



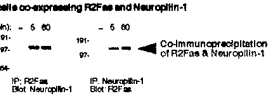
7. Over-expression of Neuropilin-1 (another VEGF receoptor) protects DU145 cells from R2Fas apoptosis



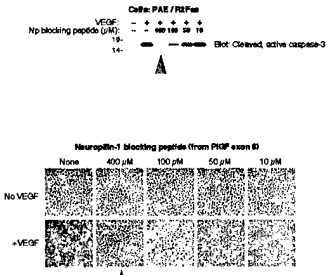
Inhibition of R2Fas-mediated apoptosis by co-expression of a second VEGF receptor (Neuropilin-1) demonstrates that R2Fas-mediated apoptosis is due to endogenous VEGF expression.

RESULTS

8. R2Fas forms a complex with Neuropilin-1 in a VEGF-dependent manner

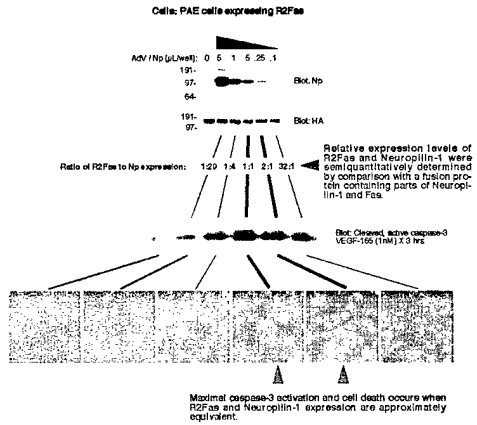


9. A peptide that blocks VEGF binding to Neuropilin-1 inhibits R2Fas apoptosis



Inhibition of R2Fas-mediated apoptosis by a Neuropilin-1 blocking peptide indicates that Neuropilin-1 contributes to the apoptotic activity of R2Fas.

10. Neuropilin-1 potentiates the activity of R2Fas when expressed at equivalent levels of expression, but over-expression of Neuropilin-1 inhibits R2Fas



Maximal caspase-3 activation and cell death occurs when R2Fas and Neuropilin-1 expression are approximately equivalent.

SUMMARY

The chimeric receptor R2Fas combines the extracellular and transmembrane domains of VEGFR-2 with the cytoplasmic death domain of the Fas apoptosis receptor.

VEGF kills endothelial cells (HUVEC or PAE) expressing R2Fas. No apoptosis occurs in the absence of VEGF.

Therefore R2Fas is a VEGF-triggered death receptor.

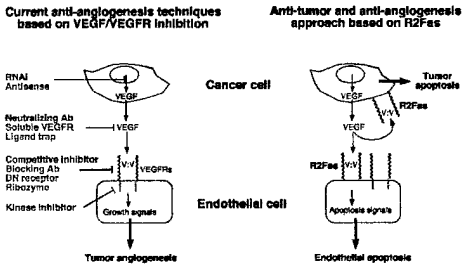
Cells that over-express VEGF (e.g. DU145 prostate cancer cells) are killed by R2Fas expression.

R2Fas stimulates apoptosis by activating Fas-mediated caspase signaling.

R2Fas forms a complex with the Neuropilin-1 receptor in a VEGF-dependent manner. Neuropilin-1 potentiates the apoptotic activity of R2Fas when expressed at similar levels.

IMPLICATIONS

These results suggest a new approach to cancer therapy in which VEGF produced by cancer cells is made to act as a death factor for the cancer cells themselves and/or their blood vessel cells.



Compared to conventional anti-angiogenesis approaches that focus on inhibiting VEGF/VEGFR expression or activity, a method based on R2Fas may have unique advantages:

- **Targets both the tumor and its vessels**
- **Kills instead of inhibiting growth**
- **Low toxicity to normal cells that express or are exposed to low levels of VEGF**
- **Increased VEGF expression in ischemic tumor and stromal cells would likely enhance R2Fas activity**

AACR American Association for Cancer Research

NATIONAL CANCER INSTITUTE

EORTC European Organization for Research and Treatment of Cancer

AACR-NCI-EORTC International Conference
Molecular Targets and Cancer Therapeutics:
Discovery, Biology, and Clinical Applications
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Contact: Warren Froelich/AACR
215/440-9300 (office)
215/341-6717 (cell)
froelich@aacr.org
Michele Baer/Feinstein Kean Healthcare
212/880-5213 (office)
917/584-9293 (cell)
michele.baer@fkhealth.com
In Boston: (11/17-11/21)
Room 203, Hynes Center
617/954-2908

Scientists Report on Promising New Approaches to Induce Cancer Cell Suicide

BOSTON (November 20, 2003) – In cancer, the normal process by which cells die is defective. Researchers are reporting today on three new approaches to activate cancer cells' ability to commit suicide. In one set of studies, scientists employed a series of molecular and gene therapy tricks to convert an angiogenic protein used by tumors to promote their growth into a cancer cell killer. In separate study, scientists used a fragment of genetic material to disable a protein used by many tumor types as an "off switch" to prevent cell death, triggering cancer cell suicide. A third study focused on a novel inhibitor to improve responses to central nervous system tumor therapy. The findings were presented today at the International Conference on Molecular Targets and Cancer Therapeutics organized by the American Association for Cancer Research (AACR), National Cancer Institute (NCI) and European Organisation for Research and Treatment of Cancer (EORTC) in Boston.

Antisense inhibition of survivin expression as a cancer therapeutic: Abstract 324
Scientists have inhibited cancer in mice using a synthetic fragment of genetic material to block production of a protein that many tumor types use to survive. Researchers at Eli Lilly and Company reported the studies of the compound, LY2181308, which is directed against a molecular target called survivin.

Survivin belongs to a family of proteins, called Inhibitor of Apoptosis Proteins, which play a key role in the regulation of apoptosis and cell division. The protein is expressed in a majority of human cancers but not in normal adult tissues, making it a potential target for cancer therapies. LY2181308 is an anti-sense oligonucleotide that potently downregulated survivin expression in human cancer cells derived from lung, colon, breast, prostate, ovary, cervix, skin and brain.

"We believe that the use of antisense molecules against targets such as survivin are a viable option for the treatment and management of cancer patients, either as a single agent or in combination with chemotherapeutic agents," said Bharvin Patel, Ph.D., of Eli Lilly, and lead investigator of the study.

In the mouse studies, the researchers tested LY2181308 against survivin in xenograft tumor model using human melanoma cells implanted under the skin. Intravenous administration of the survivin antisense molecule LY2181308 to tumor-bearing nude mice significantly inhibited tumor growth compared to animals treated with saline or mis-match control oligonucleotide. Furthermore, they demonstrated that this anti-tumor activity was associated with significant inhibition of survivin expression in these xenograft tumors. Other studies are currently assessing the activity of LY2181308 and chemotherapeutic agents in the cancer model.

Killing cancer cells and endothelial cells with a VEGF-triggered cell death receptor: Abstract 407

Scientists today presented new research on inducing cancer cell suicide. Tumors stimulate new blood vessel growth in order to acquire oxygen and nutrients, a process called tumor angiogenesis, by secreting an angiogenic growth factor called VEGF. Researchers from the University of California, San Francisco today presented a novel treatment approach using the tumor's own weapon against itself by forcing VEGF to act as a cell death factor instead of a growth factor.

VEGF normally works by attaching to the extracellular region of VEGF receptor 2, which activates the intracellular region of the receptor to send growth signals. The research team created an artificial VEGF receptor, called R2Fas, in which the intracellular region of VEGF receptor 2 was replaced with a part of the Fas death receptor, which can trigger a process of cellular suicide termed apoptosis.

Blood vessel cells in culture normally grow when exposed to VEGF. When the R2Fas receptor was expressed in blood vessel cells, the cells were instead rapidly killed by VEGF, showing that VEGF acted as a death factor instead of a growth factor. When the R2Fas receptor was expressed in cancer cells in culture that over-express VEGF, the R2Fas receptor caused the cells to die by apoptosis.

"The ability of the R2Fas receptor to switch the function of VEGF from a growth factor to a death factor may allow a new approach to anti-angiogenesis by simultaneously targeting both the VEGF-producing cancer cells and the tumor blood vessels," said Tim Quinn, M.D., assistant professor at the University of California, San Francisco Pediatrics Department. "Animal studies in which R2Fas will be expressed in tumors and tumor blood vessels will determine the feasibility of this approach."

ZD6474, a vascular endothelial growth factor reception 2 (VEGFR-2) inhibitor, inhibits growth of multiple primary central nervous system tumor types:
Abstract 709

Researchers have shown that a new and experimental drug – ZD6474 -- strongly inhibited the growth of three deadly brain tumors in animals. In the study, conducted at Duke University Medical Center, ZD6474 inhibited the growth of three types of human tumors grown in mice -- glioblastoma, medulloblastoma, and ependymoma – a remarkable finding, given that brain tumors are very distinct in their biologic makeup.

"Despite our best efforts in the laboratory and the clinic, the survival rate for glioblastoma -- the most common and lethal brain tumor -- hasn't changed in ten years," said Jeremy Rich, M.D., assistant professor in the Brain Tumor Center at Duke. "This new drug candidate has demonstrated great promise in treating human tumors that were grown in mice, and we feel these results are indicative of how the drug may act in humans."

Mice that received ZD6474 showed a 10- to 25-day delay in growth in all of the tumors compared to control mice. The drug worked by blocking tumor angiogenesis, the process by which tumor cells grow new blood vessels. Specifically, ZD6474 blocked activation of the receptor for vascular endothelial growth factor (VEGF). VEGF is a protein that malignant cells secrete in order to grow and maintain their blood vessels. When the VEGF receptor is not activated, the tumor's blood supply is diminished and the tumor shrinks and slows its spread.

Secondarily, ZD6474 prevented activation of the EGF (epidermal growth factor) receptor, another protein that cells use to grow new blood vessels, to resist dying, and to invade other cells. Additionally, ZD6474 showed promise in treating tumors that had genetically manipulated themselves to resist the effects of toxic chemotherapy.

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Founded in 1907, the American Association for Cancer Research is a professional society of more than 21,000 laboratory, translational, and clinical scientists engaged in cancer research in the United States and in more than 60 other countries. AACR's mission is to accelerate the prevention and cure of cancer through research, education, communication, and advocacy. This work is carried out through five major peer-reviewed scientific journals and high-quality scientific programs focusing on the latest developments in all areas of cancer research.

The National Cancer Institute, founded in 1971, is the principal United States government agency charged with coordinating the National Cancer Program. It facilitates international cooperation in clinical trials involving U.S. and foreign collaborating institutions.

The European Organisation for Research and Treatment of Cancer was organized in 1962 to conduct, develop, coordinate and stimulate laboratory and clinical research in Europe, and to improve the management of cancer and related problems by increasing the survival and quality of life for patients.